ENGINEERED ANIMAL TISSUE

CROSS-REFERENCES TO RELATED APPLICATIONS

Not applicable.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

Not applicable

FIELD OF THE INVENTION

This invention pertains to the field of artificial animal tissue.

BACKGROUND OF THE INVENTION

A major obstacle to studying cellular aspects of a variety of systems in humans is the lack of an appropriate and controllable model system. For example, it would be especially beneficial to have a system for studying angiogenesis. Angiogenesis is a crucially important component of embryonic development, normal physiological processes such as functions of the female reproductive tract, responses to traumatic events such a wound healing and recovery from ischemia, and pathological situations such as tumor development. Therefore, it is crucial to have an appropriate culture system that satisfactorily approximates the angiogenic environment in vivo. Studies of wound healing, cancer treatment, skin development, skin repair, skin replacement, and skin pharmatoxicology would also benefit from appropriate model systems.

Most studies to understand these types of biological processes have been conducted with animal models or with cell lines in two-dimensional cultures. Recently, three dimensional tissue culture systems have been developed that form endothelial cords with lumen (Black, et al., FASEB Journal (1998) 12:1331-1340). However, these systems are not self-maintaining and require the constant input of non-physiological factors such as phorbol esters (Montesano et al., 1983). Clearly a need exists for a three-dimensional human culture system that mimics connective tissue.

In addition, a need exists for autologous, in vitro reconstructed tissue for grafting to patients with severe trauma, e.g. nonhealing wounds or extensive burns. Early surgery and wound coverage by skin grafts have been shown to lead to better survival rates in such patients.

Currently available sources of tissue for grafting include the classic options of cadaver skin and xenografts which have serious drawbacks including lack of permanence (Berthod, F. and Rouabhia, M. (1997) Exhaustive review of clinical alternatives for damaged skin replacement. In Skin Substitute Production by Tissue Engineering: Clinical and Fundamental Applications (Rouabhia, M., editor), pp 23-45, Landes Bioseciences, Austin). Recent attempts at grafting using engineered tissue have had limited success. Unpredictable viability due to delayed vascularization of the tissue is a likely problem (Berthod, F. and Damour, O., Br. J. Dermatol. (1997) 136:809-816; Boyce, S.T., et al., J. Invest. Dermatol. (1995) 104: 345-349; Young, D.M., et al.,. J. Burn Care Rehabil. (1996) 17:305-310). Therefore, it would be beneficial to develop an engineered skin equivalent with functional vessels.

SUMMARY OF THE INVENTION

The invention provides an artificial tissue including a support matrix, microvascular endothelial cells from a first animal, and connective tissue cells from a second animal, wherein the artificial tissue includes one or more microvessels produced by the artificial tissue. In preferred embodiments, the artificial tissue further includes epithelial cells from a third animal. Most preferably, the epithelial cells form a mutlilayered epithelium.

In one embodiment, the first, second and third animals from which the artificial tissue cells are derived are mammals. Preferably, the mammals are selected from the group consisting of primate, mouse, pig, cow, cat, goat, rabbit, rat, guinea pig, hamster, horse, or sheep. Most preferably, the mammals are humans. In one embodiment, the first, second and third animals are the same. In another embodiment, the first, second and third animals are different.

In one embodiment, the support matrix of the artificial tissue of the invention includes Vitrogen®. In another embodiment, the microvascular endothelial cells of the artificial tissue of the invention include primary human adult lung microvascular cells. In still another embodiment the connective tissue cells include primary adult human dermal fibroblasts. In a further embodiment, the epithelial cells of the artificial tissue include primary human adult keratinocytes. In one preferred embodiment, the invention provides an artificial tissue including Vitrogen®, primary human adult lung microvascular cells, and primary human dermal fibroblasts, wherein the artificial tissue includes one or more microvessels produced by the artificial tissue. In another preferred embodiment, the invention provides an artificial tissue including Vitrogen®, primary human adult lung microvascular cells, primary human dermal

fibroblasts, and primary human keratinocytes, wherein the artificial tissue includes one or more microvessels produced by the artificial tissue.

In one embodiment, the artificial tissue of the invention is maintained in vitro. In a preferred embodiment, the artificial tissue of the invention is a composition suitable for tissue grafting.

The artificial tissue of the invention displays one or more characteristics of natural tissue. For example, the artificial tissue produces one or more compounds selected from the group consisting of laminin, fibronectin, collagen I, collagen III, hyaluronic acid, VEGF 145, VEGF 121, bFGF, IL-8, Syndecan-1, CXCR-1, CXCR-2, a mannose-containing protein, an acetylglucosamine-containing protein, PECAM-1, alpha-SMA, MMP-2, a growth factor receptor, plasminogen activator, mSRA, and CD68. In another embodiment, the microvessels of the artificial tissue produce one or more blood cells. In a preferred embodiment, the microvessels of artificial tissue produces one or more mononuclear leukocytes. In another embodiment, the artificial tissue produces one or more perioendothelial cells. In a further embodiment, the artificial tissue produces an extracellular matrix. In yet a further embodiment, the artificial tissue is self-maintained.

The invention also provides a method for producing an artificial tissue that entails mixing together a support matrix and connective tissue cells to form a support matrix-connective tissue mixture and forming a culture including two layers of support matrix-connective tissue mixture separated by a layer of endothelial cells, wherein the endothelial cells contact inner surfaces of the support matrix-connective tissue mixture layers. The invention includes the artificial tissue produced by this method. In a preferred embodiment, the above described method for producing an artificial tissue further includes plating a layer of epithelial cells on an outer surface of one layer of support matrix-connective tissue mixture. The invention includes the artificial tissue produced by said preferred method.

The invention further provides a method for studying a biological process, e.g., angiogenesis, wound healing, cancer treatment, skin development, skin repair, skin replacement, cell-cell interactions, cell-ECM interactions and skin pharmatoxicology. The method entails administering a test compound to the artificial tissue of the invention and measuring the effect of the test compound on a parameter of the biological process.

BRIEF DESCRIPTION ON THE FIGURES

Figure 1 is a schematic illustrating the three dimensional culture system.

Figure 2 is a set of optical microscope pictures of three-dimensional culture cross sections, illustrating microvessels with lumen and perioendothelial cells. Figure 2A is a section through a multilayered epithelium. Figure 2B is a section through microvessels surrounded by numerous fibroblasts.

Figure 3: The artificial tissue was treated with increasing concentrations of IL-8, and phosphorylation of the adhesive junction molecules PECAM-1 and VE-Cadherin was observed using immunoblots and antibodies to phosphor tyrosine (Anti-P-Y), PECAM-1 (Anti-PECAM-1), and VE-Cadherin (Anti-VE-Cadherin).

Figure 4: The artificial tissue was treated with increasing concentrations of IL-8, and production and activity of MMP-9 was measured in cell extracts and supernatant using gelatin zymogram assays.

Figure 5: The artificial tissue was treated with increasing concentrations of IL-8, and production and activity of MMP-9, TIMP-1, and TIMP-2 were measured using immunoblot analysis.

Figure 6: The artificial tissue was treated with increasing concentrations of IL-8, and activity of plasminogen activator was measured using an enzymatic assay specific for this enzyme.

DETAILED DESCRIPTION

A novel artificial tissue has been created that produces microvessels. The artificial tissue is created using a support matrix, connective tissue cells, and microvessel endothelial cells to form a three-dimensional tissue culture that exhibits at least one characteristic of connective tissue. In further embodiments, the artificial tissue is created using a support matrix, connective tissue cells, microvessel endothelial cells, and epithelial cells to form a three dimensional tissue culture that exhibits at least one characteristic of skin tissue.

The artificial tissue forms one or more microvessels with a continuous basal lamina; this is an advantage of the invention. In various embodiments, these microvessels are characterized by any of a variety of structures and/or biochemical markers, e.g., the presence of perioendothelial cells, the presence of blood cells, the presence of endothelial cell specific proteins.

Another advantage of the invention is that the artificial tissue is stably maintained and therefore does not require the addition of non-physiological factors such as tumor promoters, e.g., phorbol esters and the like. An additional advantage of some embodiments of the artificial tissue is that the tissue may be created using primary cells. A further advantage is that the artificial tissue may be created using primary human cells. Due to these advantages, the artificial tissue is particularly useful for studying various biological processes, e.g., angiogenesis. In addition the artificial tissue is useful for tissue grafts for use in patients with skin trauma such as non-healing wounds or burns.

DEFINITIONS

Terms used in the claims and specification are defined as set forth below unless otherwise specified.

For the purposes of this invention, an "artificial tissue" is a non-naturally occurring composition including at least one cell type and a supporting material.

A "support matrix" is a supporting material suitable for use in cell culture. An example of a support matrix is Vitrogen (Cohesion Technologies, Inc.).

A "primary cell" is a cell taken directly from a living organism, which cell is not immortalized.

As used herein, the term "microvascular endothelial cells" refers to cells that are from, or are derived from (e.g., after passaging), the single layer of thin flattened cells that line blood vessels. One example of microvascular endothelial cells is human primary microvascular endothelial cells from lung.

The term "connective tissue cells" refers to cells that are from, or are derived from, connective tissue. Connective tissue is a mesodermally derived, fibrous tissue, and includes tissue which holds together the cells of an organ, and tissue which surround muscles and blood vessels, etc.. In a preferred embodiment, connective tissue refers to tissue that rich in extracellular matrix and surrounds other more highly ordered tissues and organs. Examples of connective tissue cells include, but are not limited to, dermal fibroblasts.

The term "epithelial cells" refers to cells that are from or are derived from, epithelium. The epithelium is the covering of internal and external surfaces of the body, including the lining of body cavities and the skin. Examples of epithelial tissue cells include, but are not limited to, keratinocytes.

A "multilayered epithelium" is a structure that includes more than one layer of epithelial cells.

The term "microvessel" as used herein, refers to a tubular structure with one or more characteristics of any type of natural blood vessel (e.g., an artery, a vein, and the like) found in, for example, connective tissue. A microvessel can include a "junction" wherein a second microvessel has sprouted off of a first microvessel. As used herein, a microvessel includes a continuous basal lamina.

A "basal lamina" is sheet of extracellular matrix molecules characteristically found under and immediately adjacent to epithelial or endothelial cells. In one embodiment a basal lamina is characterized by the production of compounds such as collagen type IV, laminin, entactin, etc.. A "continuous basal lamina" is intended to include an uninterrupted sheet of basal lamina that surrounds a microvessel as detected by, e.g., confocal microscopy visualization of a continuous sheet of anti-laminin antibodies bound to microvessels.

The term "extracellular matrix" or "ECM" refers to material with one or more characteristics of the non-cellular material secreted by connective tissue cells into the surrounding medium. Characteristic ECM material includes fibrous elements, e.g., collagen III (ColIII), link proteins, e.g., fibronectin (FN), and laminin (LN), and space filling molecules, e.g., glycosaminoglycans.

As used herein, the term "self-maintained" means that a cell culture survives and/or grows for prolonged periods of time (e.g., ten days or more) without the addition of additional non-physiological factors such as tumor promoters, e.g., phorbol esters and the like.

For the purposes of this invention, the term "blood cells" includes cells with one or more characteristics of cells from the blood stream, e.g., white blood cells, red blood cells, or platelets. These characteristics are well-known to one of skill in the art and may be morphological, physiological or biochemical.

The term "mononuclear leukocytes" refers to cells with one or more characteristics of mononuclear leukocytes, e.g., the cell expresses CD68. These characteristics are well-known to one of skill in the art and may be morphological, physiological or biochemical.

The term "perioendothelial cells" refers to cells with one or more characteristics of perioendothelial cells. These characteristics are well-known to one of skill in the art and may

be morphological, physiological or biochemical. One example of said characteristics is expression of alpha smooth muscle actin (alpha-SMA).

As used herein, a biological "process" is an activity or function or series of activities or functions that is to be studied by a user. An example of a biological process is angiogenesis.

A "parameter" of a biological process is at least one aspect or feature of the biological process that can be observed or measure. For example, when studying the biological process of angiogenesis, one parameter that can be observed or measured is MMP-9 activity.

For the purposes of this invention, a "test compound" is any compound that is applied to the artificial tissue in order to examine the effect of the test compound on a parameter of a biological process. The compound may be natural or synthetic, purified or in a mixture. One example of a test compound is the chemokine IL-8.

The term "suitable for tissue grafting" means that an artificial tissue is in a format that is appropriate for attempting transplantation or implantation into a living organism.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

The artificial tissue

As described in more detail below, a three dimensional artificial tissue is produced using a support matrix, connective tissue cells and microvascular endothelial cells. The artificial tissue produces at least one microvessel that preferable include a continuous basal lamina. In one embodiment, the artificial tissue also includes epithelial cells which may form a multi-layered epithelium.

The cells used to produce the invention may be derived from any animal, including, but not limited to, non-mammalian vertebrates (e.g., avian, reptile and amphibian) and mammals. In a preferred embodiment, the animal cells are mammalian cells and are derived from a mammal including, but not limited to, a primate, a mouse, a pig, a cow, a cat, a goat, a rabbit, a rat, a guinea pig, a hamster, a horse, or a sheep. Primate cells are the preferred cells of the invention for many embodiments and include, for example, cells from humans, monkeys, orangutans, and baboons. In a preferred embodiment, the cells are human cells.

In one embodiment, the artificial tissue includes cells that are a mixture of cells from a plurality of species or animal. For example, the cells may be a mixture human and non-

human cells. In one embodiment, the artificial tissue includes a mixture of human and primate cells. Alternatively, the artificial tissue includes cells that are all from the same species or animal. In a preferred embodiment, the cells are all human cells.

The cells used to produce the artificial tissue may be adult cells. Alternatively, the cells used may be non-adult cells, e.g. fetal, neonatal, umbilical, etc.. Said cells may be all adult, or all non-adult, or a mixture thereof. In a preferred embodiment, the artificial tissue includes adult cells.

The cells can be from, or derived from normal tissue or abnormal tissue (i.e., tissue characterized by some physiological disorder, such as cancer). The cells can be primary cells or can be immortalized cells, e.g. altered cells with the ability to indefinitely reproduce. The cells may or may not be genetically altered. The preferred cells of the invention are primary cells from normal tissue.

To produce the artificial tissue, connective tissue cells are suspended in a support matrix to form a connective tissue-support matrix mixture. Examples of connective tissue cells suitable for use in the invention include dermal fibroblasts, pulmonary fibroblasts, mammary fibroblasts, bone marrow fibroblasts, etc.. The choice of cell type is determined by the use of the tissue, e.g., which biological process is to be studied, and/or what type of tissue grafting is to be performed. In a preferred embodiment, the connective tissue cells are normal human dermal fibroblasts.

The support matrix is a supporting material suitable for cell cultures. A support matrix can include only natural components, only synthetic components, or both natural and synthetic components. In one embodiment, the support matrix includes carbon nanotubes to facilitate flow of electrical currents to facilitate wound healing. Tensile strength of the support matrix should be suitable for the intended application, e.g., a stronger support matrix is preferred for skin grafting. Examples of support matrices are well known to one of skill in the art and include bovine collagen I and III (e.g., Vitrogen (Cohesion Technologies, Inc.), and rat collagen. In a preferred embodiment the support matrix is Vitrogen.

The support matrix is generally formed by mixing the support matrix component(s) with a tissue culture medium suitable for growth of connective tissue cells, which includes a variety of commercially available media. Examples of such media are well known to one of skill in the art and include, e.g., 1X 199 medium.

The artificial tissue is formed by co-culturing microvascular endothelial cells between two layers of the connective tissue-support matrix mixture described above. Examples of microvascular endothelial cells include but are not limited to mammary microvascular cells, umbilical microvascular cells, dermal microvascular cells, and lung microvascular cells. Again, the choice of cell type depends on the use of the tissue, e.g., which biological process is to be studied, and/or what type of tissue grafting is to be performed. In one embodiment the endothelial cells are adult human lung microvascular cells.

In a preferred embodiment, the artificial tissue is formed as follows. Vitrogen is prepared in 1X 199 medium with 0.33 M NaOH at 4°C. Normal human dermal fibroblasts are evenly suspended by mixing the fibroblasts with the Vitrogen solution to form a homogeneous connective tissue-support matrix mixture. An aliquot of this connective tissue-support matrix mixture is applied to a tissue culture growth substrate, e.g., a tissue culture well, and incubated at 37°C for 30 minutes. During this time, the mixture gels and forms the first layer of connective tissue-support matrix mixture. Primary microvascular endothelial cells from adult human lung are applied to the top of the first layer of connective tissue-support matrix mixture. Following incubation to allow attachment of the endothelial cells, a second layer of connective tissue-support matrix mixture is applied on top of the endothelial cells. An incubation period allows the second layer to gel.

In a further embodiment, the artificial tissue is formed by co-culturing microvascular endothelial cells between two layers of the connective tissue-support matrix mixture, as described above, and co-culturing epithelial cells on top of one layer of the connective tissue-support matrix mixture. Types of epithelial cells that may be used include, e.g., bladder epithelial cells, lung epithelial cells, stomach epithelial cells, and intestinal epithelial cells. The choice of cell type depends on the use of the tissue, e.g., which biological process is to be studied, and/or what type of tissue grafting is to be performed. In one embodiment, the epithelial cells are adult human keratinocytes. In a preferred embodiment, the artificial tissue is produced by co-culturing primary microvascular endothelial cells from adult human lung between two layers of a Vitrogen-primary adult normal human dermal fibroblast mixture, and co-culturing primary adult normal human keratinocytes on top of one layer of the Vitrogen- primary adult normal human dermal fibroblast mixture.

The artificial tissue of the invention can be grown in a variety of media that are well known to one of skill in the art. In particular, the culture medium contains appropriate nutrients and growth factors for the host cell employed. The nutrients and growth factors are, in many cases, well known or can be readily determined empirically by those skilled in the art. Suitable culture conditions for mammalian cells, for instance, are described in Mammalian Cell Culture (Mather ed., Plenum Press 1984); Barnes and Sato (1980) Cell 22:649; Mammalian Cell Biotechnology: a Practical Approach (Butler ed., IRL Press (1991). In one embodiment, more than one media is used at different stages of forming the artificial tissue, with an appropriate media used for each cell type, i.e., connective tissue, microvascular endothelial, and epithelial. In a preferred embodiment the media used include 1X EGM2-MV (Endothelial Growth Media 2, Microvascular, Clonetics), 1X FGM-2 (Fibroblast Growth Media 2, Clonetics), and 1X KGM-2 (Keratinocyte Growth Media 2, Clonetics), etc.. Supplements may or may not be used. In one embodiment, 5% FBS (fetal bovine serum) is included in the growth media. One of skill in the art will appreciate that the media used may be optimized for growth of different cell types.

A wide range of formats can be used for growing the artificial tissue, including, but not limited to, cell culture bottles, flasks, dishes, and multi-well plates. An optimal parameter for the growth of epithelial cells, e.g., keratinocytes, is exposure to air. Therefore, in a preferred embodiment, the artificial tissue of the invention is grown using inserts, e.g., Transwell units, Biocoat cell culture control inserts, and the like as shown schematically in Figure 1. In one embodiment, the artificial tissue is grown in 24 well plates with Biocoat cell culture control inserts (BD Biosciences). In another embodiment, the artificial tissue is grown in Transwell units (Becton Dickinson) with polyethylene tetraphthalate filters containing 8 micrometer pores.

In a preferred embodiment, the artificial tissue of the invention is self-maintaining, and as such does not require the addition of non-physiological compounds such as tumor promoters, e.g., phorbol esters.

Characterization of the artificial tissue

The artificial tissue of the invention comprises one or more structures and/or properties that are characteristic of natural tissue but have not been observed previously in artificial tissue. These structures and/or properties, as described in more detail below, can be identified by a wide range of techniques well known to one of skill in the art. The techniques may be morphological, physiological, and/or biochemical. Among the techniques are

microscopy (electron, confocal, optical, Nomarski interference), immunolabelling, immunoblotting, northern blotting, ribonuclease protection assay (RPA), reverse transcriptase polymerase chain reaction, (RT-PCR), enzyme assays, e.g., enzyme linked immunoabsorbant assays (ELISAs), zymograms, and the like.

For example, one or more microvessels are produced in the artificial tissue of the invention. The microvessels are generally composed of a tight monolayer of endothelial cells surrounding a lumen. In one embodiment, the microvessels are contacted by sparse perioendothelial cells as observed by, e.g., optical microscopy. The perioendothelial cells can also be identified by cell specific markers. In one embodiment the perioendothelial cells are identified by the cell specific marker alpha-smooth muscle actin (alpha-SMA) using immunolabelling with alpha-SMA specific antibodies and confocal microscopy.

The microvessels produced by the artificial tissue of the invention are generally surrounded by a continuous basal lamina. Among the techniques that are used to detect the continuous basal lamina are electron microscopy to visualize the basal lamina morphology, confocal microscopy coupled to immunolabelling to detect basal lamina specific components (e.g., using anti lamina-specific antibodies), and biochemistry to isolate basal lamina specific molecules, etc.. In one embodiment the continuous basal lamina is detected by confocal microscopy visualization of anti-laminin antibodies completely encircling microvessels of the artificial tissue.

In another embodiment, the microvessels present in the artificial tissue of the invention include junctions. The presence of the microvessel junctions can be confirmed using any of several microscopy techniques to visualize the microvessel morphology (e.g., phase contrast, Nomarski interference), immunolabelling to detect microvessel junction-specific components (e.g., Factor VIII), biochemistry to detect microvessel junction specific molecules such as platelet endothelial cell adhesion molecule (PECAM-1), etc.. In a preferred embodiment the microvessel junctions are identified using immunoblots to identify production of PECAM-1.

The microvessels of the artificial tissue of the invention may also contain one or more blood cells. The cells may be identified by morphological methods, immunological methods, or both. In one example, the blood cells are identified by two markers for mononuclear leukocytes, CD68 and monocyte scavenger receptor (mSRA) using immunolabelling and confocal microscopy.

Another novel feature of the artificial tissue of the invention is the production of one or more compounds characteristic of dermal tissue. Among these types of compounds are ECM molecules, growth factors and cytokines, cell surface proteins, and the like. Among the ECM molecules that can be produced by artificial tissues of the invention are fibronectin (FN), collagen III (ColIII), collagen I (ColI), proteoglycans and hyaluronic acid. In one embodiment, the artificial tissue produces at least one of FN, ColIII, ColI, a proteoglycan, and hyaluronic acid. In another embodiment, the artificial tissue produces two or more of the following ECM molecules: FN, ColIII, ColI, a proteoglycan, and hyaluronic acid. In a further embodiment, the artificial tissue produces three or more of the following ECM molecules: FN, ColIII, ColI, a proteoglycan, and hyaluronic acid. In a still further embodiment, the artificial tissue produces four or more of the following ECM molecules: FN, ColIII, ColI, a proteoglycan, and hyaluronic acid. In a preferred embodiment, the artificial tissue of the invention produces all of the following ECM molecules: FN, ColIII, ColI, a proteoglycan, and hyaluronic acid.

Among the growth factors and cytokines that can be produced by the artificial tissue of the invention are VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and interleukin- 8 (IL-8). In one embodiment, the artificial tissue produces at least one of VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8. In another embodiment, the artificial tissue produces at least two of VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8. In still another embodiment, the artificial tissue produces at least three of VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8. In a further embodiment, the artificial tissue produces at least four of VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8. In a still further embodiment, the artificial tissue produces at least five of VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8. In an alternative embodiment, the artificial tissue produces at least six of VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8. In another alternative embodiment, the artificial tissue produces at least seven of VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8. In a preferred embodiment, the artificial tissue produces all of the following: VEGF 168, VEGF 145, VEGF 121, bFGF, EGF, PDGF, IGF, and IL-8.

Among the cell surface proteins that can be produced by the artificial tissue of the invention are syndecan-1, CXCR-1, CXCR-2, cadherins, CAMs, growth factor receptors (e.g., bFGF receptor) and various mannose/n-acetylglucosamine containing proteins. In one

embodiment, the artificial tissue produces at least one of syndecan-1, CXCR-1, CXCR-2, a cadherin, a CAM, a growth factor receptor, and a mannose/n-acetylglucosamine containing protein. In another embodiment, the artificial tissue produces at least two of syndecan-1, CXCR-1, CXCR-2, a cadherin, a CAM, a growth factor receptor, and a mannose/n-acetylglucosamine containing protein. In still another embodiment, the artificial tissue produces at least three of syndecan-1, CXCR-1, CXCR-2, a cadherin, a CAM, a growth factor receptor, and a mannose/n-acetylglucosamine containing protein. In a further embodiment, the artificial tissue produces at least four of syndecan-1, CXCR-1, CXCR-2, a cadherin, a CAM, a growth factor receptor, and a mannose/n-acetylglucosamine containing protein. In a still further embodiment, the artificial tissue produces at least five of syndecan-1, CXCR-1, CXCR-2, a cadherin, a CAM, a growth factor receptor, and a mannose/n-acetylglucosamine containing protein. In an alternative embodiment, the artificial tissue produces at least six of syndecan-1, CXCR-1, CXCR-2, a cadherin, a CAM, a growth factor receptor, and a mannose/n-acetylglucosamine containing protein. In a preferred embodiment, the artificial tissue produces all of the following: syndecan-1, CXCR-1, CXCR-2, a cadherin, a CAM, a growth factor receptor, and a mannose/n-acetylglucosamine containing protein.

Uses of the artificial tissue

The artificial tissue of the invention is an excellent model for studying tissue development and repair, e.g. angiogenesis, wound healing, and tumor progression. Such studies can include evaluation of cell-cell interactions and cell-ECM interactions. The artificial tissue of the invention can also be used to study skin pharmacology, i.e., the sensitization and irritation of dermal tissue in response to a test compound.

In one embodiment, the artificial tissue of the invention is used to measure the effect of a test compound on any aspect of tissue development and repair. In a preferred embodiment, the artificial tissue of the invention is used to study angiogenesis. For example, the artificial tissue of the invention is used to study the role of cytokines in various aspects of angiogenesis including basal lamina integrity, cell adhesion, cell proliferation, cell migration, and microvessel tortuosity/sprouting. In an exemplary, preferred embodiment, the artificial tissue is used to study the role of the human chemokine IL-8 in basal lamina integrity. In a further, exemplary, preferred embodiment, the artificial tissue is used to study the role of the human chemokine IL-8 in basal lamina integrity by measuring the effect of IL-8 treatment on

artificial tissue phosphorylation of VE-cadherin and PECAM-1, production and/or activity of Matrix Metalloproteinase 9 (MMP-9), and activation of Plasminogen Activator.

The artificial tissue of the invention can also be used to develop a new-generation tissue replacement product. For example, the artificial tissue may be used to treat a variety of skin trauma, including, e.g., nonhealing wounds. In one embodiment, the artificial tissue is produced in a format suitable for skin grafting. In a preferred embodiment, the artificial tissue is created using allogenic cells. Methods for testing and using tissue suitable for skin grafting can be found, e.g., in US Patent 5,512,475.

EXAMPLE 1: FORMATION OF THE THREE DIMENSIONAL ARTIFICIAL TISSUE

A support matrix-connective tissue mixture was produced using the following steps. An interstitial support matrix of collagens I and III (Vitrogen from Cohesion Technologies) was prepared at 4°C, containing 10X 199 medium, 0.33M NaOH and 3.0 mg/ml Vitrogen at a 2:1:17 ratio by volume. Primary adult normal human dermal fibroblasts (NHDF, Clonetics, Inc., Catalogue No. CC-2511; Lot No: 16503) were passaged twice using standard tissue culture techniques. Briefly, the cells were plated at a density of $1X10^6$ cells/ml for 2 days at 37 °C and 5% CO_2 in normal plastic culture plates. The second passage of cells from the original stock were trypsinized as follows. Cells were washed twice with a glucose buffer (25 mM glucose, 140 mM NaCl, 5 mM KCl, 7 mM Na_2HPO4, pH 7.4). Trypsin (0.05% trypsin, 0.53 mM EDTA) was applied at RT. Cells were observed over time under the microscope to observe rounding up indicating release. The plates were tapped to release the single cells, and the trypsin was stopped with cold 1X media. The cells were centrifuged to a pellet (3000rpm for 3 min) and resuspended in fresh 37°C medium and then plated.

Using the trypsinized cells, 6X104 NHDF cells (~10 microliter volume in 1X FGM-2 medium, from Clonetics) were evenly suspended in 300 microliters of the collagen mixture and applied to either normal 24-well plates or 8 micrometer Biocoat cell culture control inserts (BD Biosciences). This first layer of support matrix-connective tissue mixture was incubated at 37°C for 30 minutes.

After the mixture formed a gel, microvascular endothelial cells were applied as follows. Primary adult normal microvascular endothelial cells from human lung (hMVEC, Catalogue No. CC-2527; Lot No: 7F0629) were prepared as described above for connective

tissue cells. After trypsin treatment, 1.5X10^5 hMVEC in 300 microliters of 1X EGM2-MV medium (Clonetics) were applied on top of the gel and incubated for four hours at 37°C. Attachment of the hMVECs was confirmed by microscopic visualization.

The medium was removed and a second layer of support matrix-connective tissue mixture was applied on top of the hMVEC layer as described above and incubated for 30 minutes at 37°C. Finally, EGM2-MV medium was applied on top of the artificial tissue culture (1ml of media in the 24 well plates, or 1 ml to the bottom chamber and 0.5 ml to the upper chamber in the Biocoat control inserts). Cultures were maintained at 37°C and of 5% CO2 for at least 2 weeks. Medium was changed 24 hours after the cultures were started and then fed every 48 hours.

EXAMPLE 2: FORMATION OF THE THREE DIMENSIONAL ARTIFICIAL TISSUE RESEMBLING SKIN

The artificial tissue as described in Example 1 was used as the starting material for producing an artificial tissue including epithelial cells. The artificial tissue was prepared using the Biocoat control inserts. Primary adult normal human epidermal keratinocytes (NHEK, Catalogue No. CC-2507; Lot No: 8F0538, Clonetics) were passaged twice as described above. The cells were applied (2X10^5 NHEK in 500microliters 1X KGM-2 medium (Clonetics) to the second layer of support matrix-connective tissue mixture. Media was added to both chambers of the Biocoat as follows: 1ml of EGM2-MV was added to the lower chamber and 500 microliters of KGM-2 was added to the upper chamber. Media was changed 24 hours after the NHEK were added. This feeding was followed 48 hours later by removal of the KGM-2 media from the upper chamber to allow air exposure to the NHEK. The EGM2-MV in the lower chamber was changed every 48 hours.

Figure 2 shows representative pictures of the artificial tissue resembling skin, using optical microscopy and standard techniques (e.g., Wheater PR, Burkitt HG, Daniels VG (1987) Functional histology. A Text and Color Atlas. 2nd ed. Churchill Livingstone, New York). Figure 2A is a section through a multilayered epithelium: the cell nuclei are blue and the cytoplasm is green. Like in real skin, the basal surface (b) contains the cells that divide, giving rise to the cells that progressively move up in the layer and eventually slough off at the apical surface (a). Figure 2B is a section through microvessels surrounded by numerous fibroblasts.

EXAMPLE 3: CHARACTERIZATION OF THE THREE DIMENSIONAL ARTIFICIAL TISSUE

The artificial tissue was characterized using a variety of standard techniques including microscopy and immunolabelling, northern blots, zymography, and enzyme assays.

Materials: All primary antibodies used for system characterization were specific for human antigen and were obtained from the following suppliers: anti-PECAM-1 (R&D system), anti-VE-Cadherin (Chemicon), anti-Laminin (Zymed), anti-Fibronectin (Santa Cruz Biotechnology), anti-Collagen III (Chemicon), anti-Tenascin (Chemicon), anti-Syndecan-1 (Serotec), anti-alpha-SMA (Sigma), anti-CD68 (DAKO), anti-mSRA (human macrophage scavenger receptor, kind gift from Dr. Motohito Takeya, Japan), anti-IL-8 (Intergen), anti-VEGF (Santa Cruz Biotechnology), anti-bFGF (Santa Cruz Biotechnology), anti-MMP-9 (Chemicon), anti-TIMP1 (Chemicon), and anti-TIMP2 (Chemicon). Secondary antibodies and reagents used were: anti-mouse and anti-rabbit horseradish peroxidase (Amersham: Piscataway, NJ), anti-rabbit and anti-mouse Alexa488 (Molecular Probes), anti-goat-FITC (Zymed) and anti-mouse Cy3 (Jackson ImmunoResearch). Other materials used include: biotinylated-HA binding protein (Seikagaku), WGA-Texas Red, ConA-Texas Red, Strepavidin-FITC, Strepavidin-Texas Red, nuclear staining dye TO-PRO3 (Molecular Probe), plasminogen activator substrate (CalBiochem), ECL reagents (Amersham), Vectashield mounting medium (Vector Laboratories), and DC protein assay kit (Bio-Rad).

Cells cultures: All cells, tissue culture media and materials for culture were purchased from Clonetics. Human primary microvascular endothelial cells (hMVEC), normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) were used to create three dimensional artificial tissue cultures described above in Example 2. The cultures were set up in Transwell units (Biocoat control inserts, Becton Dickinson) with polyethylene tetraphthalate filters containing 8 μm pores. Cultures were maintained at 37°C and of 5% CO_2 for at least 2 weeks.

Preparation of sections for immunolabelling: Ten-day-old cultures were fixed in 4% paraformaldehyde for 1-2 hours, washed in phosphate buffered saline (PBS) and incubated in 0.1M glycine-PBS for 10 minutes to quench free aldehyde groups. The samples were rinsed with PBS, and then incubated with 15% sucrose followed by 30% sucrose at 4°C for cryoprotection. The specimens were embedded in Tissue Freezing Medium (Triangle

Biochemical Sciences, Durham, N. C) and frozen on dry ice. Each sample was cut into 10 micrometer to 25 micrometer sections and collected in gelatin-coated slides.

Immunolabelling of sections: Sections were rinsed with PBS to remove the Tissue Freezing Medium, refixed in 4% paraformaldehyde for 10 minutes, incubated in PBS containing 0.1M glycine for 10 minutes, followed by blocking with 10% non-immune serum of the species in which the secondary antibodies were generated. Primary antibodies were used at manufacturers recommended dilutions. Primary antibodies in 1% bovine serum albumin (BSA) in PBS were applied to the sections for 1 hour at room temperature (RT) or overnight at 4°C, washed extensively with 0.1% BSA/PBS. The sections were incubated in secondary antibody for 1 hour at RT. This was followed by staining with the nuclei dye TO-PRO3 (1:1000 dilution in PBS with 0.1% Triton-X100) at RT for 10-20 minutes. After extensive washes in PBS the sections were mounted in Vectashield.

Microscopy: Immunofluorescence was imaged using a Zeiss LSM510 microscope. 3D image reconstruction was performed with the LSM510 AIM software (Zeiss).

Protein sample preparation: Cultures used for protein extraction to perform immunoblots were prepared at 4°C in 1X RIPA (containing 300mM NaCl, protease inhibitors and phosphatase inhibitor cocktail I &II (Sigma)). Cultures used for protein extraction to perform zymograms did not include inhibitors. The cultures from each well were released from the edge of culture insert and transferred to a 1.5 ml tube with 500 microliters extraction buffer with sterile forceps. The tubes were vortexed 10 times for one-half minute each time, with a 3 minute interval on ice between each vortexing step. Then tubes were left on a Nutator orbital shaker overnight at 4°C. The samples were centrifuged at 10,000rpm for 10 minutes, and the supernatants were collected and stored at -70°C until used.

Immunoblotting: SDS-PAGE was performed using 10% separating Doucet gels (Doucet, J., and J. Trifaro. A discontinous and highly porous sodium dodecyl sulfate-polyacrylamide slab gel system of high resolution. Anal. Biochem. (1988) 168:265-271.). Protein transfer to nitrocellulose membranes was performed using a wet-transfer apparatus (Bio-Rad) at 100V for 45min at room temperature. The membranes were blocked for 1 hour in 5% BSA in TTBS (0.05% Tween in Tris buffered saline) and incubated overnight at 4°C in primary antibody diluted in 1% BSA in TTBS. The membranes were washed 3 times for 20min each with TTBS, incubated in anti-mouse or anti-rabbit HRP (1:10,000) in 1% BSA for 1hr, and

washed 3 times for 20 minutes each with TTBS. The antibody bound to the membrane was visualized using ECL chemiluminescent kit (Amersham).

Zymography: Zymography was performed using 10% denaturing polyacrylamide gels as previously described (Paech and Christiansen. (1994) In Cell biology: a laboratory handbook. (J. Celis, Ed.) V.3 pp.264-271 San Diego: Academic Press). Briefly, gels to test for gelatinolytic activity contained 1% gelatin (Sigma) and were cast 12 hours before the sample separation. Protein samples were separated at 4°C with 10mA constant current for each gel. The proteins were renatured using two washes of 15 minutes each in 2.5% Triton-X100 twice at RT. Renaturation solution was replaced by developing buffer (50mM Tris-HCl, 200mM NaCl, 10mM $CaCl_2$, 0.02% Brij 35, pH7.5), the gels were incubated at 37°C for 8-14 hours, fixed (methanol:water:acetic acid, 5:5:1) for 10 minutes, stained with 0.5% Coomassie Blue for 1 hour and destained (40% methanol plus 10% acetic acid) to obtain appropriate contrast.

Results:

Data and results are shown in Appendix A.

EXAMPLE 4: USING THE THREE DIMENSIONAL ARTIFICIAL TISSUE TO STUDY ANGIOGENESIS

The most common process by which new blood vessels form in adults occurs by sprouting from pre-existing vessels (angiogenesis). This process can be productively divided into the following steps: (1) Destabilization of endothelium, leading to blood vessel permeability and flexibility; (2) degradation of the basal lamina that surrounds the preexisting blood vessels; (3) localized proliferation of endothelial cells to provide cells for sprout formation; (4) endothelial cell migration, tube formation and elongation of the sprout; (5) stabilization of the endothelium by deposition of new basal lamina; (6) recruitment of perioendothelial support cells to the endothelium; (7) stabilization of the interactions between perioendothelial cells and endothelial cells; and (8) formation of mature microvessels. Numerous factors have been identified that participate in these steps of sprout formation during embryonic development. However, in adults, angiogenesis driven by injury or pathological conditions involves additional/alternative factors, including chemokines, of which IL-8 is a major player (e.g. Arenberg et al., 1997a & b).

It has been observed that the homologue of human IL-8 (hIL-8) in chickens, cCAF (chicken Chemotactic and Angiogenic Factor), stimulates angiogenesis in the

Chorioallantoic Membrane (CAM) assay and in young chicks (Martins-Green and Feugate, 1998). Because of the high homology between cCAF and hIL-8 it is possible that the human chemokine also stimulates blood vessel sprouting. The artificial tissue of the invention was used to investigate the role of the test compound IL-8 on angiogenesis.

Cell cultures: Three dimensional artificial tissues were created as described above in Example 1 using primary normal human microvascular endothelial cells from adult lung(hMVEC; Clonetics), and primary adult normal human dermal fibroblasts (NHDF; Clonetics). The cultures were set up in either 24-well plates or Transwell units (Becton Dickinson) with polyethylene tetraphthalate filters containing 4 μm pores. Cultures were maintained at 37°C and of 5% CO_2 for at least 2 weeks. Medium was changed 24 hours after the cultures were started and then fed every 48 hours.

Immunoblotting: SDS-PAGE was performed using 10% separating Doucet gels (Doucet, J., and J. Trifaro. A discontinous and highly porous sodium dodecyl sulfate-polyacrylamide slab gel system of high resolution. Anal. Biochem. (1988) 168:265-271.). Protein transfer to nitrocellulose membranes was performed using a wet-transfer apparatus (Bio-Rad) at 100V for 45min at room temperature. The membranes were blocked for 1 hour in 5% BSA in TTBS (0.05% Tween in Tris buffered saline) and incubated overnight at 4°C in primary antibody diluted in 1% BSA in TTBS. The membranes were washed 3 times for 20min each with TTBS, incubated in anti-mouse or anti-rabbit HRP (1:10,000) in 1% BSA for 1hr, and washed 3 times for 20 minutes each with TTBS. The antibody bound to the membrane was visualized using ECL chemiluminescent kit (Amersham).

Zymography: Zymography was performed using 10% denaturing polyacrylamide gels as previously described (Paech and Christiansen. (1994) In Cell biology: a laboratory handbook. (J. Celis, Ed.) V.3 pp.264-271 San Diego: Academic Press). Briefly, gels to test for gelatinolytic activity contained 1% gelatin (Sigma) and were cast 12 hours before the sample separation. Protein samples were separated at 4°C with 10mA constant current for each gel. The proteins were renatured using two washes of 15 minutes each in 2.5% Triton-X100 twice at RT. Renaturation solution was replaced by developing buffer (50mM Tris-HCl, 200mM NaCl, 10mM $CaCl_2$, 0.02% Brij 35, pH7.5), the gels were incubated at 37°C for 8-14 hours, fixed (methanol:water:acetic acid, 5:5:1) for 10 minutes, stained with 0.5% Coomassie Blue for 1 hour and destained (40% methanol plus 10% acetic acid) to obtain appropriate contrast.

Plasminogen activity assay: A chromogenic plasminogen activator substrate (Boc-Val-Gly-Arg- NA.AcOH) was purchased from Calbiochem. 50µl of a 10mM stock solution of substrate in water was added to 200µl of 0.1mM HEPES buffer pH 8.0 and heated to 25°C. 25µl cell extract was added to the substrate and absorbance was then read at 405nm.

Results:

Figure 3 illustrates the results from an experiment where the artificial tissue was treated with increasing concentrations of IL-8, and phosphorylation of the adhesive junction molecules PECAM-1 and VE-Cadherin was observed using immunoblots. The artificial tissue (one well in duplicate for each IL-8 concentration) was treated with IL-8 at the following concentrations (in ng/ml): 50, 100, 300, 600, and 1000. The samples were treated for 30 minutes and cell extracts were prepared, electrophoresed, and blotted. Membranes were probed with antibodies to phosphor tyrosine (Anti-P-Y), PECAM-1 (Anti-PECAM-1), and VE-Cadherin (Anti-VE-Cadherin). The results demonstrate that hIL-8 stimulates the phosphorylation of VE-cadherin and PECAM in microvessels. Therefore, IL-8 probably plays a role in destabilization of the endothelium reflected in loss of adhesion between the endothelial cells.

Figure 4 illustrates the effects of IL-8 on production and activation of MMP-9 in the artificial tissue. Gelatin zymogram assays were performed to detect the production and activity of MMP-9. IL-8 increases MMP-9 production and activation in a dose-dependent manner. This effect was observed both in the Cell/ECM extracts and in the culture medium (supernatants). Samples were collected 24 hours after IL-8 treatment.

Figure 5 illustrates the effects of IL-8 on MMP-9 and related natural tissue inhibitors. The artificial tissue was treated with increasing concentrations of IL-8, and production and activity of MMP-9 was measured using immunoblot analysis. The same blots were also probed for the MMP-9 natural tissue inhibitors TIMP-1 and TIMP-2. Samples were collected 3 hours after IL-8 treatment.

Figure 6 illustrates the effects of IL-8 on activation of Plasminogen Activator. The artificial tissue was treated with increasing concentrations of IL-8, and activity of plasminogen activator was measured using an enzymatic assay specific for the enzyme.

Additional data and results are in Appendix B.

While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this

disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques and apparatus described above can be used in various combinations. All publications, patents, patent applications, and/or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent application, and/or other document were individually indicated to be incorporated by reference for all purposes.

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